Method for Producing Target Substance by Fermentation

Field of the Invention

The present invention relates to a technique used in the fermentation industry, more precisely, a method for efficiently producing a target substance such as L-amino acids by fermentation utilizing a microorganism.

Description of the Related Art

Bacterial cells have been modifying their metabolic pathways, respiratory pathways and so forth in order to adapt to various environments. In the energy metabolism, Arc (aerobic respiration control) and Fnr (fumarate nitrate reduction) are known as control systems playing important roles. These consist of global regulator proteins and universally existing in *E. coli* and other analogous species. The former is encoded by the *arcA* gene existing at the position of 0 minute of the *E. coli* chromosome, the latter is encoded by the *fnr* gene existing at the position of 29 minutes of the *E. coli* chromosome, and the both adapt the cell to an environment by controlling many factors under an anaerobic condition. Moreover, it has been elucidated that the ArcA protein and the Fnr protein are transcription factors, and they positively or negatively control expression of a target gene on the *E. coli*

chromosome under an anaerobic condition by directly binding to a promoter region of the target gene (S. Iuchi et al., Cell, 66, 5-7 (1991)).

Recently, expression profiles of strains in which genes coding global regulators such as the ArcA protein and Fnr protein derived from *E. coli* are disrupted are collected in a database by using DNA microarray techniques and opened to the public (http://www.genome.ad.jp/dbget-bin/get_htext?Exp_DB+-n+Bget-bin/get_htext?Exp_DB+-n+B).

So far, it is known that the ArcA protein negatively controls expression of the genes for the tricarboxylic acid cycle (S. Iuchi et al., Cell, 66, 5-7 (1991)), and the expression of the genes for the tricarboxylic acid cycle is increased in the *arcA*-disrupted strain in the database. On the other hand, it is known that the Fnr protein positively controls gene expression for the respiratory pathway that functions under an anaerobic condition.

As for the expression profiles in the global factor-disrupted strains, the *dam*-disrupted strain can be mentioned as a strain in which gene expression for the TCA is increased like the *arcA*-disrupted strain (H. Mori, Nara Institute of Science and Technology, oral announcement at the symposium "Green Biotechnology of Genome Age", 2001, organized by Japan Bioindustry Association, Resource Biotransformation Study Group).

The Dam protein is a methylase for modification factors involved in intracellular restriction modification systems, and it is encoded by the *dam* gene existing at the position of 76 minutes of the *E. coli* chromosome (Proc. Natl. Acad. Sci. U.S.A., 87 (23), 9454-9458 (1990)).

It has not been reported so far about improvement of substance production through expression control of the global factors such as genes *arcA*, *fnr* and *dam*.

Description of the Related Art

An object of the present invention is to improve production efficiency in production of a useful substance by fermentation utilizing a γ-proteobacterium such as *Escherichia* bacteria.

The inventors of the present invention conducted various researches in order to achieve the aforementioned object, and they found production of substance by a γ-proteobacterium could be improved by modifying a gene coding for a regulator protein universally existing in γ-proteobacteria. That is, they found that an ability to produce a target substance could be improved by disrupting the *arcA* gene in a γ-proteobacterium and thus accomplished the present invention.

That is, the present invention provides followings.

(1) A γ-proteobacterium having an ability to produce a

target substance and modified so that an ArcA protein does not normally function.

(2) The γ-proteobacterium according to (1), wherein the ArcA protein that normally functions is a protein defined in the following (A) or (B):

(A) a protein having the amino acid sequence of SEQ ID NO: 32;

(B) a protein having the amino acid sequence of SEQ ID NO: 32 including substitution, deletion, insertion or addition of one or several amino acids and improving an ability to produce a target substance when the protein does not normally function in the γ-proteobacterium compared with the case where the protein normally functions.

(3) The γ-proteobacterium according to (1), wherein the ArcA protein that normally functions is a protein having 70% or more of homology to the amino acid sequence of SEQ ID NO: 32 and improving an ability to produce a target substance when the protein does not normally function in the γ-proteobacterium compared with the case where the protein normally functions.

(4) The γ-proteobacterium according to (1), wherein the ArcA protein that normally functions is a protein having the amino acid sequence of SEQ ID NO: 32 including substitution, deletion, insertion or addition of 2 to 20 amino acids and improving an ability to produce a target substance when the protein does not normally function in

the γ-proteobacterium compared with the case where the protein normally functions.

(5) The γ-proteobacterium according to any one of (1) to (4), wherein the ArcA protein does not normally function by means of disruption of an *arcA* gene on a chromosome.

(6) The γ-proteobacterium according to (5), wherein the *arcA* gene is DNA defined in the following (a) or (b):

(a) DNA containing the nucleotide sequence of the nucleotide numbers 101 to 817 of SEQ ID NO: 31;

(b) DNA hybridizable with the nucleotide sequence of the nucleotide numbers 101 to 817 of SEQ ID NO: 31 or a probe that can be produced from the nucleotide sequence under the stringent condition and coding for a protein that improves an ability to produce a target substance when the protein does not normally function compared with the case where the protein normally functions.

(7) The γ-proteobacterium according to any one of (1) to (6), which is a bacterium belonging to the genus *Escherichia*.

(8) The γ-proteobacterium according to any one of (1) to (7), wherein the target substance is an L-amino acid.

(9) The γ-proteobacterium according to (8), wherein the L-amino acid is selected from the group consisting of L-lysine, L-glutamic acid and L-arginine.

(10) A method for producing a target substance, which comprises culturing the γ-proteobacterium according to

any one of (1) to (9) in a medium to produce and accumulate the target substance in the medium or cells and collecting the target substance from the medium or cells.

According to the present invention, when a useful substance such as L-amino acids is produced by using a γ-proteobacterium, the production efficiency can be improved.

Brief Explanation of the Drawing

Fig. 1 shows accumulation patterns in WC196, WC196ΔarcA, WC196Δdam and WC196Δfnr.

Detailed Explanation of the Invention

Hereafter, the present invention will be explained in detail.

<1> γ-Proteobacterium of the present invention

The γ-proteobacterium used for the present invention is not particularly limited so long as it is a microorganism belonging to γ-proteobacteria such as genus *Escherichia*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, *Erwinia*, *Salmonella*, *Morganella* or the like and has an ability to produce a target substance. Specifically, those classified into the γ-proteobacteria according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database

(http://www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg?mode=Tree&id=1236&lvl=3&keep=1&srchmode=1&unlock) can be used.

Examples of the bacterium belonging to the genus *Escherichia* include *E. coli* and so forth. Examples of the belonging to the genus *Enterobacter* include *Enterobacter agglomerans*, *Enterobacter aerogenes* and so forth.

There are some species of *Enterobacter agglomerans* recently re-classified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii agglomerans* or the like based on nucleotide sequence analysis of 16S rRNA etc. In the present invention, the bacterium may belong to either the genus *Enterobacter* or *Pantoea* so long as it is classified into γ-proteobacteria and has the *arcA* gene.

When *E. coli* is bred by using genetic engineering techniques, the *E. coli* K12 strain and derivatives thereof can be used. Further, when *Pantoea ananatis* is bred by using genetic engineering techniques, *Pantoea ananatis* strains AJ13355 (FERM BP-6614), AJ13356 (FERM BP-6615) and AJ13601 (FERM BP-7207), and derivatives thereof can be used. Although the above-mentioned strains were identified as *Enterobacter agglomerans* when they were isolated, these strains has been re-classified into *Pantoea ananatis* based on nucleotide sequence analysis of 16S rRNA etc. as described above.

The γ-proteobacterium of the present invention is any one of the aforementioned bacteria, and is a bacterium having an ability to produce a target substance. The "ability to produce a target substance" means an ability to produce and accumulate the target substance in cells or a medium in such a degree that, when the bacterium of the present invention is cultured in the medium, the target substance can be collected from the cells or medium.

The target substance to be produced according to the present invention is not particularly limited, so long as it is a substance that is produced by a γ-proteobacterium and synthesized via the tricarboxylic acid cycle or a substance synthesized from such a substance as a substrate. Examples include, for example, those conventionally produced by γ-proteobacteria, i.e., various amino acids such as L-lysine, L-threonine, L-isoleucine, L-glutamic acid, L-glutamine and L-arginine, organic acids such as L-homoserine and succinic acid and so forth. Further, the present invention can also be applied to a substance that has not so far been industrially produced by using γ-proteobacteria, so long as it can be synthesized from a substance synthesized via the TCA cycle as a substrate.

As L-lysine producing γ-proteobacteria, there can be exemplified mutants having resistance to an L-lysine analogue. This L-lysine analogue is a substance that

inhibits growth of L-amino acid producing strain, but this inhibition is fully or partially canceled when L-lysine coexists in a medium. Examples of the L-lysine analogue include oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (AEC), γ-methyllysine, α-chlorocaprolactam and so forth. Mutants having resistance to these lysine analogues can be obtained by subjecting γ-proteobacteria to a conventional artificial mutagenesis treatment. Specific examples of bacterial strain used for producing L-lysine include *E. coli* AJ11442 (FERM BP-1543, NRRL B-12185; refer to Japanese Patent Laid-open Publication (Kokai) No. 56-18596 and U.S. Patent No. 4,346,170) and *E. coli* VL611. In these microorganisms, feedback inhibition of aspartokinase by L-lysine is desensitized.

In addition to the above, there can be mentioned, for example, L-threonine producing bacteria described later, because inhibition of aspartokinase by L-lysine is generally eliminated also in L-threonine producing bacteria.

In the Examples described later, the WC196 strain was used as an L-lysine producing bacterium of *E. coli*. This bacterial strain was bred by imparting AEC resistance to the W3110 strain derived from *E. coli* K-12. This strain was designated as the *E. coli* AJ13069, and was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and

Technology (presently, the independent administrative corporation, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, postal code: 305-8566, Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on December 6, 1994 and received an accession number of FERM P-14690. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and received an accession number of FERM BP-5252 (refer to International Patent Publication WO96/17930).

Examples of L-threonine producing γ-proteobacteria include *E. coli* VKPM B-3996 (RIA 1867, refer to U.S. Patent No. 5,175,107), strain MG442 (refer to Gusyatiner et al., Genetika (in Russian), 14, pp.947-956, 1978) and so forth.

Examples of the microorganism belonging to γ-proteobacteria and having L-glutamic acid producing ability include, for example, microorganisms deficient in α-ketoglutarate dehydrogenase activity or having reduced α-ketoglutarate dehydrogenase activity. Bacteria belonging to the genus *Escherichia* deficient in α-ketoglutarate dehydrogenase activity or having reduced α-ketoglutarate dehydrogenase activity and methods for obtaining them are described in Japanese Patent Laid-open Publication (Kokai) Nos. 5-244970 and 7-203980. Specifically, the following strains can be mentioned.

E. coli W3110sucA::Km^r

E. coli AJ12624 (FERM BP-3853)

E. coli AJ12628 (FERM BP-3854)

E. coli AJ12949 (FERM BP-4881)

E. coli W3110sucA::Km^r is a strain obtained by disrupting the α-ketoglutarate dehydrogenase gene (hereinafter referred to as "*sucA* gene") of *E. coli* W3110, and it is a strain completely deficient in the α-ketoglutarate dehydrogenase.

Microorganisms belonging to γ-proteobacteria and deficient in α-ketoglutarate dehydrogenase activity or having reduced α-ketoglutarate dehydrogenase activity and methods for obtaining them are described in Japanese Patent Laid-open Publication (Kokai) Nos. 5-244970 and 7-203980.

Examples of L-arginine producing γ-proteobacteria include *E. coli* into which *argA* gene has been introduced (Japanese Patent Laid-Open Publication No.57-5693) and *E. coli* strain 237 (Russian Patent No. 200117677) or the like.

Examples of L-isoleucine producing γ-proteobacteria include *E. coli* KX141 (VKPM B-4781, refer to European Patent Laid-open Publication No. 519,113).

Examples of L-homoserine producing *Escherichia* bacteria include the NZ10 strain, which is a Leu^+ revertant of the C600 strain (refer to Appleyard R.K., Genetics, 39, pp.440-452, 1954).

As succinic acid producing γ-proteobacteria, examples using *E. coli* are known (Wang, X., et al., Appl. Biochem. Biotech., 70-72, 919-928 (1998)).

Further, bacteria belonging to the genus *Escherichia* having L-amino acid producing ability can also be bred by introducing DNA having genetic information involved in biosynthesis of L-amino acids and enhancing the ability utilizing a gene recombination technique. For example, as for L-lysine producing bacteria, examples of genes that can be introduced include, for example, genes coding for enzymes of the biosynthetic pathway of L-lysine such as phosphoenolpyruvate carboxylase, aspartokinase, dihydrodipicolinate synthetase, dihydrodipicolinate reductase, succinyldiaminopimelate transaminase and succinyldiaminopimelate deacylase. In case of a gene of an enzyme suffering from feedback inhibition by L-aspartic acid or L-lysine such as phosphoenolpyruvate carboxylase or aspartokinase and dihydrodipicolinate synthetase, it is desirable to use a mutant gene coding for an enzyme in which such inhibition is eliminated.

Further, as for L-glutamic acid producing bacteria, examples of genes that can be introduced include genes of glutamate dehydrogenase, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase, phosphoenolpyruvate carboxylase, pyruvate dehydrogenase, pyruvate kinase,

phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, fructose bis-phosphate aldolase, phosphofructokinase, glucose phosphate isomerase and so forth.

Further, an activity of an enzyme that catalyzes a reaction for producing a compound other than the target L-amino acid by branching off from the biosynthetic pathway of the L-amino acid may be decreased or made deficient. For example, examples of such an enzyme that catalyzes a reaction for producing a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine include homoserine dehydrogenase (refer to International Patent Publication WO95/23864). Further, examples of an enzyme that catalyzes a reaction for producing a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid include α-ketoglutarate dehydrogenase, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrophosphate dehydrogenase and so forth.

In breeding of γ-proteobacteria having such a target substance producing ability as mentioned above, to introduce a gene into γ-proteobacteria to enhance

their ability, there can be used a method in which a vector autonomously replicable in a γ-proteobacterium cell is ligated to the gene to produce recombinant DNA and γ-proteobacterium is transformed with it. In addition, it is also possible to incorporate a target gene into host chromosome by a method using transduction, transposon (Berg, D.E. and Berg, C.M., Bio/Technol. 1, p.417, 1983), Mu phage, (Japanese Patent Laid-open Publication (Kokai) No. 2-109985) or homologous recombination (Experiments in Molecular Genetics, Cold Spring Harbor Lab., 1972). Further, the target gene can also be introduced by a method of disrupting a gene using a linear DNA produced by PCR (Kirill A., Datsenko et al., Proc. Natl. Acad. Sci. USA., 97 (12), 6640-6645 (2000)).

Examples of the γ-proteobacteria bred by recombinant DNA techniques as described above include, for example, bacteria belonging to the genus *Escherichia* having enhanced activities of dihydrodipicolinate synthase having a mutation canceling feedback inhibition by L-lysine, aspartokinase, dihydrodipicolinate reductase and so forth, of which feedback inhibition by L-lysine is desensitized, and having L-lysine producing ability (U.S. Patent No. 6,040,160), and bacterium belonging to the genus *Enterobacter* (the genus *Pantoea*) having enhanced activity of citrate synthase, phosphoenolpyruvate carboxylase or glutamate

dehydrogenase and having L-glutamic acid producing ability (EP 0 952 221 A2, EP 0 999 282 A2, EP 1 078 989 A2).

The γ-proteobacterium used for the present invention is a bacterium having an ability to produce the aforementioned target substance and modified so that the ArcA protein does not normally function in a cell. The expression of "modified so that the ArcA protein does not normally function in a cell" means that it is modified so that the function of the ArcA protein should be completely eliminated, or the function should be reduced compared with an unmodified strain of *Escherichia* bacterium such as a wild strain. The state where the ArcA protein does not normally function may be, for example, a state where transcription or translation of the *arcA* gene is inhibited, and hence the gene product thereof, the ArcA protein, is not produced or the production thereof is reduced, or a state where the produced ArcA protein is mutated, and thus the proper function of the ArcA protein is reduced or eliminated. Examples of the γ-proteobacteria in which the ArcA protein does not normally function include, typically, a gene-disrupted strain in which the *arcA* gene on the chromosome is disrupted by a genetic recombination technique, and a mutant strain in which an expression regulatory sequence or coding region of the *arcA* gene on the chromosome is mutated, and therefore functional ArcA

protein is no longer produced.

Examples of the ArcA protein contained in a wild strain or unmodified strain used for the breeding of the bacterium of the present invention include, for example, a protein having the amino acid sequence of SEQ ID NO: 32. Further, examples of the *arcA* gene include, for example, DNA having the nucleotide sequence of SEQ ID NO: 31. Moreover, the gene may have the sequence in which any codon is replaced with another equivalent codon. In the present invention, the term "DNA coding for a protein" means that, when DNA is double-stranded, either one of the strands codes for the protein.

Further, the ArcA protein contained in the wild strain or unmodified strain is not limited to a wild-type protein, and it may contain substitution, deletion, insertion, addition or the like of one or more amino acid residues so long as the protein has the activity of ArcA protein. Although the number of "several" amino acid residues referred to herein differs depending on position or type of amino acid residues in the three-dimensional structure of the protein, it may be specifically 2 to 30, preferably 2 to 20, more preferably 2 to 10.

The aforementioned "activity of the ArcA protein" is an activity that improves the ability to produce a target substance when the protein does not function normally compared with the case where the protein

normally functions. In other words, the activity of the ArcA protein means that a γ-proteobacterium modified so that the protein does not normally function produces and accumulates a larger amount of the target substance in a medium compared with an unmodified strain of the γ-proteobacterium such as a wild strain. Examples of wild strain of *E. coli* include, for example, the K12 strain and derivative thereof such as *E. coli* MG1655 strain (ATCC No. 47076) and W3110 strain (ATCC No. 27325). Further, examples of unmodified strain of *Pantoea ananatis* (*Enterobacter agglomerans*) include the strains AJ13355 (FERM BP-6614), AJ13356 (FERM BP-6615) and AJ13601 (FERM BP-7207).

The aforementioned substitution, deletion, insertion, addition, inversion or the like of amino acid residues also include naturally occurring mutations or variations due to difference in individual, species, strain or the like of the microorganism containing the ArcA protein.

Examples of such mutants or variants of the *arcA* gene as mentioned above include DNA that is hybridizable with a nucleotide sequence comprising the sequence of the nucleotide numbers 101 to 817 in SEQ ID NO: 31 or a probe that can be produced from the nucleotide sequence under the stringent condition and codes for a protein having an activity similar to that of ArcA. The "stringent condition" used herein is a condition under

which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent condition is exemplified by a condition under which DNAs having high homology, for example, DNAs having homology of 50% or more, preferably 70% or more, more preferably 80% or more, are hybridized with each other, but DNAs having homology lower than the above are not hybridized with each other. More specifically, the stringent condition is exemplified by a condition under which DNAs are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C.

As the probe, a partial sequence of the nucleotide sequence of SEQ ID NO: 31 can also be used. Such a probe can be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 31 as primers and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 31 as a template. When a DNA fragment having a length of about 300 bps is used as the probe, the washing conditions for the hybridization may consist of 50°C, 2 x SSC and 0.1% SDS.

The terms *arcA* gene and ArcA protein used hereafter are not limited to those having the nucleotide sequence or amino acid sequence shown in SEQ ID NO: 31

or 32, but include mutants or homologues thereof. As an example of the homologue, the nucleotide sequence of *arcA* gene and the amino acid sequence of ArcA of *Pantoea ananatis* are shown in SEQ ID No: 19 and 20.

The bacterium of the present invention is a bacterium modified so that the ArcA protein does not normally function, specifically, a γ-proteobacterium of which *arcA* gene is disrupted, for example. Such a bacterium can be obtained by, for example, substituting an *arcA* gene that does not normally function (hereafter also referred to as "disrupted *arcA* gene") for the *arcA* gene on the chromosome by homologous recombination utilizing a genetic recombination technique (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press (1972); Matsuyama, S. and Mizushima, S., J. Bacteriol., 162, 1196 (1985)).

The mechanism of the homologous recombination is as follows. When a plasmid or the like carrying a sequence exhibiting homology with a chromosomal sequence is introduced into a corresponding bacterial cell, recombination occurs at a site of the homologous sequence at a certain frequency, and thus the introduced plasmid as a whole is integrated into the chromosome. Then, by causing recombination again at the site of the homologous sequence on the chromosome, the plasmid may be removed again from the chromosome. However, depending on the position at which the recombination is

caused, the disrupted gene may remain on the chromosome, while the original normal gene may be removed from the chromosome together with the plasmid. By selecting such a bacterial strain, a bacterial strain in which the normal *arcA* gene is replaced with the disrupted *arcA* gene can be obtained.

Such a gene disruption technique based on the homologous recombination has already been established, and a method utilizing a linear DNA, a method utilizing temperature sensitive plasmid or the like can be used therefor. The *arcA* gene can also be disrupted by using a plasmid that contains the *arcA* gene inserted with a marker gene such as drug resistance gene, and cannot replicate in a target microbial cell. That is, in a transformant that has been transformed with such a plasmid and hence acquired drug resistance, the marker gene is integrated into the chromosome DNA. It is likely that this marker gene has been integrated by homologous recombination of the *arcA* gene present at the both sides of the marker with these genes on the chromosome, and therefore a gene-disrupted strain can efficiently be selected.

Examples of temperature sensitive plasmid functioning in *Escherichia* bacteria include pMAN997 (International Patent Publication WO99/03988), pHSG415, pHSG422 (Hashimoto-Gotoh, T. et al, Gene, 16, 227-235 (1981)) and so forth.

Specifically, a disrupted *arcA* gene used for the gene disruption can be obtained by deletion of a certain region of *arcA* gene by means of digestion with restriction exzyme(s) and religation, by insertion of another DNA fragment (marker gene etc.) into the *arcA* gene, or by introducing substitution, deletion, insertion, addition or inversion of one or more nucleotides in a nucleotide sequence of coding region of *arcA* gene, its promoter region or the like by means of site-specific mutagenesis (Kramer, W. and Frits, H. J., Methods in Enzymology, 154, 350 (1987)) or treatment with a chemical reagent such as sodium hyposulfite and hydroxylamine (Shortle, D. and Nathans, D., Proc. Natl. Acad. Sci. U.S.A., 75, 270 (1978)) or the like, so that the activity of the encoded repressor should be reduced or eliminated, or transcription of the *arcA* gene should be reduced or eliminated. Among these methods, a method utilizing deletion of a certain region of the *arcA* gene by digestion with a restriction enzyme and religation, or insertion of another DNA fragment into the *arcA* gene is preferred in view of reliability and stability.

The sequence of *arcA* gene per se is known, and therefore the *arcA* gene can be easily obtained by the PCR method or hybridization method based on the sequence. It is sufficient that the *arcA* gene used for the gene disruption should have homology in such a degree that homologous recombination with the *arcA* gene contained in

the target bacterium should be caused. Specifically, it is sufficient that the homology should be usually 70% or more, preferably 80% or more, more preferably 90% or more.

Disruption of the target gene can be confirmed by analyzing the gene on the chromosome utilizing Southern blotting or PCR method.

Methods for obtaining various genes, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation etc. used for the present invention are described in Sambrook, J., Fritsch, E.F., Maniatis, T., Molecular Cloning, Cold Spring Harbor Laboratory Press, 1.21 (1989).

Further, a mutant strain in which functional ArcA protein is no longer produced can be obtained by subjecting a γ-proteobacterium to ultraviolet irradiation or treating it with a mutating agent used for usual mutation treatment such as N-methyl-N'-nitrosoguanidine (NTG) or nitrous acid.

By culturing a γ-proteobacterium microorganism having an ability to produce a target substance and modified so that the ArcA protein does not normally function, which can be obtained as described above, in a medium to produce and accumulate the target substance in the medium or cells and collecting the target substance from the medium or cells, the target substance can be produced. According to the present invention, the

production efficiency of the target substance can be improved by using a γ-proteobacterium having the aforementioned characteristics. It is estimated that the *arcA* gene is expressed in a wild strain of γ-proteobacterium concerning the *arcA* gene during the culture and inhibits the expression of the genes involved in the TCA cycle, whereas in a strain in which the ArcA protein does not normally function, such expression inhibition for the TCA cycle genes is canceled, and thus the above effect should be obtained.

The medium used for the present invention may be an ordinary medium containing a carbon source, nitrogen source, inorganic ions, and other organic components as required. As the carbon source, there can be used saccharides such as glucose, sucrose, lactose, galactose, fructose, arabinose, maltose, xylose, trehalose, ribose and starch hydrolysate, alcohols such as glycerol, mannitol and sorbitol and organic acids such as gluconic acid, fumaric acid, citric acid and succinic acid. As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate, ammonium chloride and ammonium phosphate, organic nitrogen such as soybean protein hydrolysate, ammonia gas, aqueous ammonia and so forth. As organic trace amount nutrients, it is desirable to add required substances, for example, vitamins such as vitamin B_1, nucleic acids such as adenine and RNA or yeast extract or the like to the

medium in appropriate amounts. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and so forth are added in small amounts as required.

The culture may be carried out under conventionally used well-known conditions depending on the bacterial strain used. For example, the culture is preferably carried out under an aerobic condition for 16 to 72 hours. Culture temperature is preferably controlled to be 30°C to 45°C, and pH is preferably controlled to be 4.5 to 8 during the culture. Inorganic or organic, acidic or alkaline substances as well as ammonia gas and so forth can be used for pH adjustment.

For collection of the target substance from the medium or cells, any special method is not required for the present invention. That is, it can be carried out by a combination of conventionally well-known techniques such as methods utilizing ion exchange resins, precipitation and other techniques depending on the type of the target substance. Further, the target substance accumulated in cells can be collected, after the cells are physically or enzymatically disrupted, from cell extract or membrane fraction depending on the target substance. Furthermore, depending on the target substance, cells containing the target substance can also be used as they are as a microbial catalyst or the like.

Examples

Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Disruption of arcA, *dam* and *fnr* genes of *E. coli*

The entire nucleotide sequence of genomic DNA of *E. coli* K-12 strain has been already elucidated (Blattner F.R., Plunkett G., Bloch C.A. et al., Science, 227, 1453-1474 (1997); ftp://ftp.genetics.wisc.edu/pub/sequence/ecolim52.seq.gz). Based on the known nucleotide sequences of arcA, *dam* and *fnr* genes, gene-disrupted strains for each of arcA, *dam* and *fnr* were produced. In the following procedure, QIAGEN-Genomic-tip System (produced by QIAGEN) was used for the extraction of genomic DNA.

(1) Disruption of *arcA* gene of *E. coli*

Primers were synthesized based on the reported nucleotide sequence of arcA, and N- and C-terminal fragments of *arcA* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template. Pyrobest DNA Polymerase (produced by Takara Shuzo) was used for PCR, and PCR was performed according to the attached instruction. Primers 1 and 2 were used

as the primers for PCR for amplifying N-terminal fragment, and Primers 3 and 4 were used as the primers for PCR for amplifying C-terminal fragment. Primer 1 was designed to contain a *Hin*dIII site, and Primer 4 was designed to contain an *Xba*I site.

Primer 1: cccaagcttaaagccctttacttagctta (sequence complementary to the nucleotide numbers 5482 to 5501 of the nucleotide sequence of GenBank Accession No. AE000510 added with ccc and *Hin*dIII site at the 5' end, SEQ ID NO: 1)

Primer 2: tccgcgccatctgtcgcttc (sequence of the nucleotide numbers 4851 to 4870 of the nucleotide sequence of GenBank Accession No. AE000510, SEQ ID NO: 2)

Primer 3: gaagcgacagatggcgcggaaaagctacaagttcaatggt (sequence complementary to the nucleotide numbers 4541 to 4560 of the nucleotide sequence of GenBank Accession No. AE000510 added at the 5' end with a sequence complementary to the nucleotide numbers 4851 to 4870 of the nucleotide sequence of GenBank Accession No. AE000510, SEQ ID NO: 3)

Primer 4: gggtctagaggttgaaaaataaaaacggc (sequence of the nucleotide numbers 4188 to 4207 of the nucleotide sequence of GenBank Accession No. AE000510 added with ggg and *Xba*I site at the 5' end, SEQ ID NO: 4)

After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit

(produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 1 and 4 were used for the crossover PCR method (A.J. Link, D. Phillips, G.M. Church, Journal of Bacteriology, 179, 6228-6237 (1997)) to obtain a disrupted *arcA* fragment. The purified DNA fragment was digested with *Hin*dIII and *Xba*I (produced by Takara Shuzo) and subjected to phenol/chloroform treatment and ethanol precipitation. This fragment was ligated with a temperature sensitive plasmid pMAN997 (International Patent Publication WO99/03988) also digested with *Hin*dIII and *Xba*I by using DNA ligation Kit Ver.2 (produced by Takara Shuzo). JM109 competent cells (produced by Takara Shuzo) were transformed with this ligation solution and applied to an LB agar plate containing 25 μg/mL of ampicillin (produced by Sigma) (LB + ampicillin plate). After the cells were cultured at 30°C for one day, the grown colonies were cultured in test tubes at 30°C in LB medium containing 25 μg/mL of ampicillin, and plasmids were extracted by using an automatic plasmid extractor PI-50 (produced by Kurabo Industries). The obtained plasmids were digested with *Hin*dIII and *Xba*I and subjected to agarose gel electrophoresis, and the plasmid inserted with the target fragment was designated as plasmid pMAN_ΔarcA for *arcA* disruption. The aforementioned pMAN997 is a plasmid obtained by exchanging *Vsp*I-*Hin*dIII fragments of pMAN031 (S.

Matsuyama and S.Mizushima, J. Bacteriol., 162, 1196 (1985)) and pUC19 (produced by Takara Shuzo).

The *E. coli* WC196 strain was transformed with the plasmid pMAN_ΔarcA according to the method of C.T. Chung et al., and colonies were selected on an LB + ampicillin plate at 30° C. The selected clones were cultured overnight at 30° C as liquid culture, then the culture broth was diluted to 10^{-3} concentration and plated on an LB + ampicillin plate, and colonies were selected at 42° C. The selected clones were applied to an LB + ampicillin plate and cultured at 30° C, and then 1/8 of the cells on the plate were suspended in 2 mL of LB medium and cultured at 42° C for 4 to 5 hours with shaking. The culture broth was diluted to 10^{-5} concentration and applied to an LB plate, and several hundreds of colonies among the obtained colonies were inoculated on an LB plate and LB + ampicillin plate to confirm growth and thereby select ampicillin sensitive strains. Colony PCR was performed for several ampicillin sensitive strains to confirm the deletion of *arcA* gene. In this way, an *arcA* disrupted-strain derived from *E. coli* WC196, WC196ΔarcA, was obtained.

(2) Disruption of *dam* gene of *E. coli*

A *dam* gene-disrupted strain was produced from WC196 in the same manner as in (1).

Primers were synthesized based on the reported

nucleotide sequence of the *dam* gene, and N- and C-terminal fragments of the *dam* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template. Primers 5 and 6 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 7 and 8 were used as the primers for PCR for amplifying C-terminal fragment. Primer 5 was designed to contain a *Hin*dIII site, and Primer 8 was designed to contain an *Xba*I site.

Primer 5: cccaagcttccgtggtatgtcctggtttc (sequence complementary to the nucleotide numbers 5150 to 5169 of the nucleotide sequence of GenBank Accession No. AE000414 added with ccc and *Hin*dIII site at the 5′ end, SEQ ID NO: 5)

Primer 6: agactgatcaggtcgctatt (sequence of the nucleotide numbers 4741 to 4760 of the nucleotide sequence of GenBank Accession No. AE000414, SEQ ID NO: 6)

Primer 7: aatagcgacctgatcagtctgccttatgcaccgctgtctg (sequence complementary to the nucleotide numbers 4361 to 4380 of the nucleotide sequence of GenBank Accession No. AE000414 added at the 5′ end with a sequence complementary to the nucleotide numbers 4741 to 4760 of the nucleotide sequence of GenBank Accession No. AE000414, SEQ ID NO: 7) Primer 8: gggtctagacgtcagattgggaacatagt (sequence of the nucleotide numbers 3931 to 3950 of the nucleotide

sequence of GenBank Accession No. AE000414 added with ggg and *Xba*I site at the 5' end, SEQ ID NO: 8)

After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 5 and 8 were used for the crossover PCR method to obtain a deficient type *dam* fragment. The following procedure was performed in the same manner as in (1) to obtain a *dam* disrupted-strain WC196Δdam.

(3) Disruption of *fnr* gene of *E. coli*

A *fnr* gene-disrupted strain was produced from WC196 in the same manner as in (1).

Primers were synthesized based on the reported nucleotide sequence of the *fnr* gene, and N- and C-terminal fragments of the *fnr* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template.

Primers 9 and 10 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 11 and 12 were used as the primers for PCR for amplifying C-terminal fragment. Primer 9 was designed to contain a *Hin*dIII site, and Primer 12 was designed to contain an *Xba*I site. A *fnr*-disrupted strain was produced from WC196 in the same manner as in (1).

Primer 9: cccaagcttgcaattgggccgtcctggcg (sequence

complementary to the nucleotide numbers 7981 to 8000 of the nucleotide sequence of GenBank Accession No. AE000231 added with ccc and *Hin*dIII site at the 5′ end, SEQ ID NO: 9)

Primer 10: tcaagctgatcaagctcatg (sequence of the nucleotide numbers 7501 to 7520 of the nucleotide sequence of GenBank Accession No. AE000231, SEQ ID NO: 10)

Primer 11: caggagttgatcagcttgagaaaatgccgaggaacgtc (sequence complementary to the nucleotide numbers 7121 to 7140 of the nucleotide sequence of GenBank Accession No. AE000231 added at the 5′ end with a sequence complementary to the nucleotide numbers 7501 to 7520 of the nucleotide sequence of GenBank Accession No. AE000231, SEQ ID NO: 11) Primer 12: gggtctagattggtcgtcctggttaggat (sequence of the nucleotide numbers 6671 to 6690 of the nucleotide sequence of GenBank Accession No. AE000231 added with ggg and *Xba*I site at the 5′ end, SEQ ID NO: 12)

After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 9 and 12 were used for the crossover PCR method to obtain a deficient type *dam* fragment. The following procedure was performed in the same manner as in (1) to obtain a *fnr* disrupted-strain WC196Δfnr.

Example 2: Effect of *arcA* disruption on L-lysine production in *E. coli* strain

The *arcA* gene-disrupted strain, WC196ΔarcA strain, the *dam* gene-disrupted strain, WC196Δdam, the *fnr* gene-disrupted strain, WC196Δfnr, and the parent strain thereof, WC196, were cultured, and their L-lysine production amounts were measured. The media, culture methods and analysis method for the measurement are shown below.

[Base medium: E-100 medium]

	Final concentration
Glucose	10 g/L (separately sterilized)
NH_4Cl	20 mM
$NaHPO_4$	40 mM
KH_2PO_4	30 mM
$CaCl_2$	0.01 mM
$FeSO_4$	0.01 mM
$MnSO_4$	0.01 mM
citric acid	5 mM
thiamine hydrochloride	2 mM (separately sterilized)
casamino acid	2.5 g/L (separately sterilized)
MES-NaOH (pH 6.8)	50 mM (separately sterilized)

[Culture method]

Refresh culture:

Stock bacteria were inoculated.

LB agar medium (drug was added as required), 37°C, 24 hours.

Seed culture:

The bacteria undergone the refresh culture were inoculated in a volume of 2 mL to LB medium.

LB medium (drug was added as required), 37°C, overnight.

Main culture:

1/16 of the bacteria on the seed culture cell plate were inoculated.

E-100 medium (drug was added as required), 37°C, 20 ml in 500 ml-volume Sakaguchi flask.

[Analysis method]

The culture broth was sampled in a volume of 500 μl in a time course, and glucose concentration and L-lysine accumulation in the culture broth were measured. The glucose concentration and L-lysine accumulation were measured for supernatant of the culture broth obtained after centrifugation at 15,000 rpm for 5 minutes diluted to an appropriate concentration with water by using Biotech Analyzer (Sakura Seiki). The results are shown in Fig. 1.

As a result, it was observed that the *fnr* gene-disrupted strain exhibited L-lysine accumulation equivalent to that of the control strain, and the *dam* gene-disrupted strain exhibited reduced accumulation

compared with the control strain. On the other hand, it was recognized that the L-lysine accumulation of the *arcA* gene-disrupted strain was improved compared with the control strain.

Example 3: Effect of *arcA* disruption on L-glutamic acid production in *E. coli* strain

Since L-lysine accumulation improvement effect was observed in Example 2 by the use of *arcA* gene disruption, effect of the *arcA* gene on the L-glutamic acid fermentation was examined in this example.

In order to confirm effect of deficiency of the *arcA* gene on L-glutamic acid production in *E. coli* MG1655, *E. coli* MG1655-derived *sucA* deficient strain (MG1655ΔsucA) and *E. coli* MG1655-derived *sucA* and *arcA* doubly deficient strain (MG1655ΔsucAΔarcA) were constructed.

(1) Disruption of *sucA* gene of *E. coli*

A *sucA* gene-disrupted strain was produced from MG1655 in the same manner as in Example 1.

Primers were synthesized based on the reported nucleotide sequence of the *sucA* gene, and N- and C-terminal fragments of the *sucA* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template.

Primers 13 and 14 were used as the primers for PCR

for amplifying N-terminal fragment, and Primers 15 and 16 were used as the primers for PCR for amplifying C-terminal fragment. Primer 13 was designed to contain a *Hin*dIII site, and Primer 16 was designed to contain an *Xba*I site. A sucA-disrupted strain was produced from MG1655 in the same manner as in (1).

Primer 13: cccaagcttctgcccctgacactaagaca (sequence of the nucleotide numbers 10721 to 10740 of the nucleotide sequence of GenBank Accession No. AE000175 added with ccc and *Hin*dIII site at the 5' end, SEQ ID NO: 13)

Primer 14: cgaggtaacgttcaagacct (sequence complementary to the nucleotide numbers 11501 to 11520 of the nucleotide sequence of GenBank Accession No. AE000175, SEQ ID NO: 14)

Primer 15: aggtcttgaacgttacctcgatccataacgggcagggcgc (sequence of the nucleotide numbers 12801 to 12820 of the nucleotide sequence of GenBank Accession No. AE000175 added at the 5' end with a sequence of the nucleotide numbers 10501 to 11520 of the nucleotide sequence of GenBank Accession No. AE000175, SEQ ID NO: 15)

Primer 16: gggtctagaccactttgtcagtttcgatt (sequence complementary to the nucleotide numbers 13801 to 13820 of the nucleotide sequence of GenBank Accession No. AE000175 added with ggg and *Xba*I site at the 5' end, SEQ ID NO: 16)

After PCR, the amplified DNA fragments were each

purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 13 and 16 were used for the crossover PCR method to obtain a deficient type *sucA* fragment. The following procedure was performed in the same manner as in (1) to obtain a *sucA* disrupted-strain, MG1655ΔsucA.

(2) Preparation of *sucA* and *arcA* gene doubly deficient strain of *E. coli*

In the same manner as in Example 1, the *arcA* gene of MG1655ΔsucA was disrupted to prepare a *sucA* and *arcA* doubly deficient strain (MG1655ΔsucAΔarcA).

Similarly, *sucA* and *dam* doubly deficient strain (MG1655ΔsucAΔdam) and *sucA* and *fnr* doubly deficient strain (MG1655ΔsucAΔfnr) were produced.

In order to examine effect of *arcA* gene disruption on L-glutamic acid fermentation, the doubly deficient strains for the genes, MG1655ΔsucAΔarcA, MG1655ΔsucAΔdam, and MG1655ΔsucAΔfnr strains as well as the *sucA* gene deficient strain, MG1655ΔsucA, as a control were cultured, and L-glutamic acid production amounts were measured. The media, culture methods and analysis method for the measurement are shown below.

[Base medium: MS medium]

	Final concentration
Glucose	40 g/L (separately sterilized)

$MgSO_4 \cdot 7H_2O$	1 g/L (separately sterilized)
$(NH_4)_2SO_4$	16 g/L
KH_2PO_4	1 g/L
Yeast extract	2 g/L
$FeSO_4$	0.01 g/L
$MnSO_4$	0.01 g/L
$CaCO_3$	30 g/L (separately sterilized)

[Culture methods]

Refresh culture:

Stock bacteria were inoculated.

LB agar medium (drug was added as required), 37°C, 24 hours.

Seed culture in test tube:

The bacteria undergone the refresh culture were inoculated.

LB liquid medium (drug was added as required), 37°C, 16 hours.

Main culture:

10% of the liquid medium for the seed culture was inoculated.

MS liquid medium (drug was added as required), 37°C, 20 ml in 500 ml-volume Sakaguchi flask.

[Analysis method]

The culture broth was sampled in a volume of 500 μl in a time course, and glucose concentration and L-

glutamic acid accumulation in the culture broth were measured. The glucose concentration and L-glutamic acid concentration were measured for supernatant of the culture broth obtained after centrifugation at 15,000 rpm for 5 minutes diluted to an appropriate concentration with water by using Biotech Analyzer (Sakura Seiki). The L-glutamic acid accumulation and yield at the point where the saccharide was depleted are shown in Table 1.

Table 1: L-glutamic acid accumulation and yield of *sucA* and *arcA*-disrupted strain

Strain	L-glutamic acid accumulation (g/L)	L-glutamic acid yield (%)
MG1655ΔsucA	15.4	36.9
MG1655ΔsucAΔarcA	17.0	41.7
MG1655ΔsucAΔdam	14.2	35.5
MG1655ΔsucAΔfnr	14.6	36.6

As a result, both of the accumulation and yield of glutamic acid were slightly lower in the *sucA* and *dam* gene-disrupted strain compared with the control, and they were comparable to those of the control in the *sucA* and *fnr* gene-disrupted strain. On the other hand, it was recognized that both of the accumulation and yield of L-glutamic acid were improved in the *sucA* and *arcA* gene-disrupted strain compared with the control strain.

Example 4: Disruption of *arcA* gene of *Pantoea ananatis*

<1> Acquisition of *arcA* gene of Pantoea ananatis

(1) Construction of *Pantoea ananatis* producing L-glutamic acid under a low pH condition

ArcA is a global regulator universally existing in *E. coli* and other relative species. Using a bacterium belonging to the genus *Pantoea*, *Pantoea ananatis* AJ13601, which is a relative to *E. coli*, *arcA* gene of *Pantoea ananatis* was obtained based on a known nucleotide sequence of *E. coli arcA*. The strain AJ13601 was obtained as follows (refer to EP 1 078 989 A2). The strain AJ13355 was isolated from soil in Iwata-shi, Shizuoka, Japan as a strain which can graw under a low pH in a medium containing L-glutamic acid and carbon source. From the strain AJ13355, the strain SC17 was selected as a less mucus producing mutant which shows good growth. The strain SC17sucA, in which α-ketoglutarate dehydrogenase (αKGDH) gene is disrupted, was constructed from the strain SC17. To the strain SC17sucA, the plasmid pSTVCB containing a citrate synthase gene (*gltA*) derived from *Brevibacterium lactofermentum* (pSTVCB), and the plasmid RSFCPG containing *gltA*, phosphoenolpyruvate carboxylase gene (*ppc*) and glutamate dehydrogenase gene (*gdhA*) derived from *E. coli* were introduced. From the obtained transformants, the strain AJ13601 was selected as a strain which has an increased resistance to high

concentration of L-glutamic acid under a low pH condition. The strain AJ13601 has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (presently, the independent administrative corporation, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-5466) on August 18, 1999, under accession number of FERM P-17516, and then, the deposit was converted into international deposit under the provisions of the Budapest Treaty on June 6, 2000 and received accession number of FERM BP-7207 (refer to EP 1 078 989 A2).

(2) Acquisition of *arcA* gene of *Pantoea ananatis* AJ13601

Genomic DNA of *Pantoea ananatis* AJ13601 was extracted using QIAGEN-Genomic-tip System (produced by QIAGEN). By PCR using the genomic DNA as a template and the following oligonucleotides as primers, 759bp DNA fragment containing *arcA* gene ORF was obtained. Pyrobest DNA Polymerase (produced by Takara Shuzo) was used for PCR, and PCR was performed according to the attached instruction. Primers 17 and 18 were used as PCR primers for amplification. Primer 17 was designed to contain a *Eco*RI site, and Primer 18 was designed to contain an *Sph*I site, respectively.

Primer 17: cccgaattccctgtttcgatttagttggc (sequence complementary to the nucleotide numbers 4980-4999 of the nucleotide sequence of GenBank Accession No. AE000510 added with *Eco*RI site at the 5' terminus: SEQ ID NO: 17)

Primer 18: cccgcatgcgattaatcttccagatcacc (sequence of the nucleotide numbers 4245-4264 of GenBank Accession No. AE000510 added with *Sph*I site at the 5' terminus: SEQ ID NO: 18)

The obtained DNA fragment was inserted to the cloning vector pSTV29 (produced by Takara Shuzo) in the forward direction as to the direction of transcription by *lacZ* gene utilizing *Eco*RI and *Sph*I sites designed in the primers to obtain pSTV29_EaarcA. The nucleotide sequence of the cloned sequence is shown in SEQ ID No: 19. The deduced amino acid sequence encoded by the ORF is shown in SEQ ID No: 20. The obtained ORF shows about 81.2% identity in the nucleotide sequence and about 92.1% in the amino acid sequence to the *arcA* gene of *E. coli*. Thus the ORF is considered to encode ArcA of *Pantoea ananatis*.

<2> Disrution of *arcA* gene of *Pantoea ananatis*

Pantoea ananatis strain G106S was used for construction of *arcA* gene-disrupted strain of *Pantoea ananatis*. Among the two plasmids, RSFCPG and pSTVCB, harbored by the strain AJ13601, the strain G106S harbors

RSFCPG alone and is deleted pSTVCB. The *arcA* gene-disrupted strain was constructed from the strain G106S. Then pSTVCB was introduced to the obtained gene-desrputed strain to obtain the *arcA* gene-disrupted strain of AJ13601. The procedure will be explained below in detail.

(1) Constructin of a plasmid for conjugative transfer for disruption of *arcA* gene

Utilization of conventional breeding by recombination on choromosome with a temperature-sensitive plasmid is not simple in a recombination procedure for *Pantoea ananatis* because of characteristics of *Pantoea ananatis* that it can hardly grow at 42°C. Therefore, a technique of recombination on a choromosome utilizing conjugative transfer was used in this experiment. For the conjugative transfer method, it is necessary to construct a plasmid which does not contain a replication origin (ori) of *Pantoea ananatis*, that is, a plasmid which cannot replicate *Pantoea ananatis*. Thus, the oriR6K and mobRP4 region was amplified by PCR using primers 21 and 22, and a plasmid for Tn5 transfer, pUT/miniTn5-Cm (Lorenzo V., et al., Journal of Bacteriology, 172, 6568- (1990); Herrero M., et al., Journal of Bacteriology, 172, 6557 (1990)) as a template. Besides, a fragment containing multi-cloning site and chloramphenicol resistance gene was amplified by PCR

using primers 23 and 24, and pHSG399 as a template. Each of the obtained amilified fragment was digested with *Bgl*II (produced by Takara Shuzo) and the fragments were ligated with DNA ligation Kit ver.2 (produced by Takara Shuzo).

Then, *E. coli* strain S17-1 λpir (R. Simon., et al., BIO/TECHNOLOGY NOVEMBER 1983, 784-791 (1983)) was transformed with the ligation mixture, and applied onto LB agar plate containing 30μg/ml of chloramphenicol. After culture for one day at 37°C, appeared colonies were cultured in LB medium containing 30μg/ml of chloramphenicol in test tubes at 37°C. Plasmids were obtained from each of the culture using QIAprep Mini Spin column Kit (produced by QIAGEN). Obtained plasmids were digested with *Bgl*II, and a plasmid which has a unique *Bgl*II recognition site was designated as the plasmid for conjugative transfer, pUT399Cm.

Primer 21: tcatagatcttttagattgatttatggtgc (SEQ ID NO: 21)

Primer 22: ccacagatctaattcccatgtcagccgtta (SEQ ID NO: 22)

Primer 23: ataaagatctgtgtccctgttgataccggg (SEQ ID NO: 23)

Primer 24: ggggagatcttgcaaggcgattaagttggg (SEQ ID NO: 24)

Then, a kanamycin resistance gene was introduced

into pUT399Cm and deleted the chloramphenicol resistance gene from the plasmid according to the following procedure. The kanamycin resistance gene was amplified by PCR using primers 25 and 26, and pMW 219 (produced by Nippon Gene) as a template. Pyrobest DNA Polymerase (produced by Takara Shuzo) was used for PCR, and PCR was performed according to the attached instruction. Each of primers 25 and 26 was added with *Bgl*II site at the 5' terminus. Obtained DNA fragment and pUT399 were digested with *Bgl*II (produced by Takara Shuzo), and ligated with DNA ligation Kit ver.2 (produced by Takara Shuzo). Then, *E. coli* strain S17-1 λpir (R. Simon., et al., BIO/TECHNOLOGY NOVEMBER 1983, 784-791 (1983)) was transformed with the ligation mixture, and applied onto LB agar plate containing 25μg/ml of kanamycin (LB + kanamycin plate). After culture for one day at 37°C, appeared colonies were cultured in LB medium containing 25μg/ml of kanamycin in test tubes at 37°C. Plasmids were obtained from each of the culture using QIAprep Spin Miniprep Kit (produced by QIAGEN). Obtained plasmids were digested with *Bgl*II, and subjected to agarose gel electrophoresis, and the plasmid inserted with the target fragment was designated as plasmid pUT399CmKm.

Primer 25: cccagatctagttttcgccccgaagaacg (SEQ ID NO: 25)
Primer 26: cccagatctccagagtcccgctcagaaga (SEQ ID NO: 26)

Then, the chloramphenicol resistance gene was deleted from pUT399CmKm as described below. pUT399CmKm was digested with *Hin*dIII (produced by Takara Shuzo) and was ligated with DNA ligation Kit ver.2 (produced by Takara Shuzo). *E. coli* strain S17-1 λpir (R. Simon., et al., BIO/TECHNOLOGY NOVEMBER 1983, 784-791 (1983)) was transformed with the ligation mixture, and applied onto LB agar plate containing 25μg/ml of kanamycin (LB + kanamycin plate). After culture for one day at 37° C, appeared colonies were cultured on LB agar plate containing 25μg/ml of kanamycin and LB agar plate containing 30μg/ml of chloramphenicol (produced by Sigma) at 37° C and a strain showing chloramphenicol sensitivity. The strain was cultured in LB medium containing 25μg/ml of kanamycin for one day at 37° C and plasmid was obtained from the culture using QIAprep Spin Miniprep column Kit (produced by QIAGEN). Obtained plasmids was designated pUT399km.

Primers were prepared based on the nucleotide sequence of *arcA* gene obtained in the above <1>, and N-terminal fragment and C-terminal fragment of the *arcA* gene were amplified using the primers and pSTV29_EaarcA as a template. Pyrobest DNA Polymerase (produced by Takara Shuzo) was used for PCR, and PCR was performed according to the attached instruction. Primers 27 and 28 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 29 and 30 were used as

terminal fragment, and Primers 29 and 30 were used as the primers for PCR for amplifying C-terminal fragment. Primer 27 was designed to contain an *Eco*RI site, and Primer 30 was designed to contain an *Sph*I site, respectively.

Primer 27: cccgaattcgcgaccgatggtgcagagat (SEQ ID NO: 27)
Primer 28: aaggcaaattcatggtgcgc (SEQ ID NO: 28)
Primer 29: gcgcaccatgaatttgccttacccaatgaagagcgtcgcc (SEQ ID NO: 29)
Primer 30: cccgcatgcaccttcgccgtgaatggtgg (SEQ ID NO: 30)

After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 27 and 30 were used for the crossover PCR method (A.J. Link, D. Phillips, G.M. Church, Journal of Bacteriology, 179, 6228-6237 (1997)) to obtain a disrupted *arcA* fragment.

The purified DNA fragment was digested with *Eco*RI and *Sph*I (produced by Takara Shuzo) and subjected to phenol/chloroform treatment and ethanol precipitation. This fragment was ligated with a plasmid pUT399Km also digested with *Eco*RI and *Sph*I by using DNA ligation Kit Ver.2 (produced by Takara Shuzo). *E. coli* strain S17-1 λpir (R. Simon., et al., BIO/TECHNOLOGY NOVEMBER 1983, 784-791 (1983)) was transformed with the ligation

25μg/ml of kanamycin. After culture for one day at 37°C, appeared colonies were cultured in LB medium containing 25μg/ml of kanamycin in test tubes at 37°C. Plasmid was obtained from the culture using QIAprep Spin Miniprep column Kit (produced by QIAGEN). Obtained plasmids were digested with *Eco*RI and *Sph*I, and subjected to agarose gel electrophoresis. The plasmid inserted with the target fragment was designated as plasmid pUT399Km_ΔarcA for *arcA* disruption.

(2) Disrution of *arcA* gene of *Pantoea ananatis* by conjugative transfer

Gene disruption using homologous recombination method with the above-mentioned pUT399Km_ΔarcA. The strain G106S was used as a plasmid donor strain. Screening was performed with a medium comprising 5g/L of glucose (produced by Junsei Kagaku), 5g/L of Yeast Extract (produced by Difco), 10g/L of Trypotone-Peptone (Difco), 10g/L of NaCl (Junsei Kagaku), 6g/L of Na_2HPO_4, 3g/L of KH_2PO_4, 1g/L of NH_4Cl, and 1.5g/L of $CaCl_2 \cdot 2H_2O$ (hereinafter referred to as "LBG-M9 medium") added with containing 25μg/mL of tetracycline, 25μg/ml of kanamycin and agar (hereinafter referred to as "LBG-M9+Tet+Km" plate). On the agar medium, the strain G106S into which pUT399Km_ΔarcA has been incorporated on its chromosome can be selected as a single-recombination strain, that is, *arcA* gene-disrupted strain, since the plasmid

derived from pUT399 cannot replicate in *Pantoea ananatis* as described above. *E. coli* strain S17-1 λpir (R. Simon., et al., BIO/TECHNOLOGY NOVEMBER 1983, 784-791 (1983)) was transformed with pUT399Km_ΔarcA, and applied onto LB agar plate containing 25μg/ml of kanamycin. After culture, obtained transformant, *E. coli* S17-1 λpir/pUT399Km_ΔarcA, was cultured in LBG-M9 medium containing 25μg/ml of tetracycline for one day at 37°C. Besides, the strain G106S were cultured in LBG-M9 medium containing 25μg/ml of tetracycline for one day at 34°C. The each of culture media was centrifuged and obtained cells were suspended in 50μl of LB medium, respectively. 25μl of each suspension was mixed and cultured in LBG-M9 agar medium for one hour at a room temperature. Subsequently, the culture was continued for 3 hours at 34°C to cause conjugative transfer. Then the cultured cells diluted to 10^{-1}, 10^{-2} or 10^{-3} concentration were applied to LBG-M9+Tet+Km plate and strains resistant to tetracycline and kanamycin were selected. Colony PCR was performed for some strains among the selected strains to confirm deletion of *arcA* gene. Thus, the *arcA*-disrupted strain derived from G106S, G106SΔarcA was obtained.

(3) Introduction of pSTVCB into G106SΔarcA and production of L-glutamic acid

The strain G106SΔarcA was transformed with pSTVCB. Obtained transformant G106SΔarcA/pSTVCB is equivalent to

Obtained transformant G106SΔarcA/pSTVCB is equivalent to *arcA* gene-disrupted strain of the above-mentioned AJ13601 (AJ13601ΔarcA). The strain G106SΔarcA/pSTVCB and the strain AJ13601 as a control were cultured, and their L-glutamic acid production amounts were measured, respectively. The media, culture methods and analysis method for the measurement are shown below.

[Evaluation medium for L-glutamic acid]

	Final concentration
Sucrose	30 g/L (separately sterilized)
$MgSO_4 \cdot 7H_2O$	0.5 g/L (separately sterilized)
$(NH_4)_2SO_4$	20 g/L
KH_2PO_4	2 g/L
Yeast extract	2 g/L
$FeSO_4$	0.02 g/L
$MnSO_4$	0.02 g/L
Lysine	0.2 g/L
Methionine	0.2 g/L
Diamino pimelate	0.2 g/L
pH7.0 (KOH)	
$CaCO_3$	20 g/L (separately sterilized)

[Culture methods]

Seed culture in test tube:

Stock bacteria were inoculated.

LBG-M9 agar medium (drug was added as required), 34°C, 24 hours.

Main culture:

Three platinum loops of the seed culture was inoculated.

Base medium(drug was added as required), 34°C, 24 hours.

5ml per test tube.

[Analysis method]

The culture broth was sampled in a volume of 400 μl in a time course, and sucrose concentration and L-glutamic acid accumulation in the culture broth were measured. The sucrose concentration and L-glutamic acid concentration were measured for supernatant of the culture broth obtained after centrifugation at 15,000 rpm for 5 minutes diluted to an appropriate concentration with water by using Biotech Analyzer (Sakura Seiki). The L-glutamic acid accumulation and yield at the point where the saccharide was depleted are shown in Table 2.

Table 2: L-glutamic acid accumulation and yield of *arcA*-disrupted strain

Strain	L-glutamic acid accumulation (g/L)	L-glutamic acid yield (%)
G106(AJ13601)	16.3	50.8
G106ΔarcA(AJ13601ΔarcA)	17.4	54.3

As a result, it was recognized that both of the

accumulation and yield of L-glutamic acid were improved in the *arcA* gene-disrupted strain compared with the control strain.

Example 5: Effect of *arcA* disruption on L-arginine production in *E. coli* strain

In the above Exmaple 2, it was recognized that both of the accumulation and yield of L-glutamic acid were improved in the *sucA* and *arcA* gene-disrupted strain compared with the control strain, *sucA*-disrupted strain.

Then, the effect on the production of L-arginine which is produced using glutamic acid as a substrate. E. coli strain 237 was used as an L-arginine producing strain. The strain 237 was deposited at Russian National Collection of Industrial Microorganisms (VKPM) on April 10, 2000, under accession number of VKPM B-7925, and then, the deposit was converted into international deposit under the provisions of the Budapest Treaty on May 18, 2001.

(1) Construction of *arcA* gene-disrupted strain of *E. coli* strain 237

An *arcA* gene of the strain 237 was disrupted to prepare an arcA gene-disrupted strain, 237ΔarcA in the same manner as in Example 1.

(2) Production of L-arginine

To evalutate an effect of *arcA* gene disruption on L-aniginine fermentation, *arcA* gene-disrupted strain of 237, 237ΔarcA, and the strain 237 as a control were cultured and their L-arginine production amounts were measured. The media, culture methods and analysis method for the measurement are shown below.

[Evaluation medium for L-arginine]

	Final concentration
Glucose	60 g/L (separately sterilized)
$MgSO_4 \cdot 7H_2O$	1 g/L (separately sterilized)
$(NH_4)_2SO_4$	25 g/L
KH_2PO_4	2 g/L
Yeast extract	5 g/L
Thiamine	0.1 mg/L
pH7.2	
$CaCO_3$	25 g/L (separately sterilized)

[Culture methods]

Seed culture in test tube:

Stock bacteria were inoculated.

LB agar medium (drug was added as required), 32°C, 24 hours.

Main culture:

One platinum loops of the seed culture was inoculated.

Evaluation medium for arginine (drug was added as

required), 32°C, 3 days.

2ml per test tube.

[Analysis method]

The culture broth was sampled in a volume of 500 μl in a time course, and glucose concentration and L-arginine accumulation in the culture broth were measured. The glucose concentration and the L-arginine concentration were measured for supernatant of the culture broth obtained after centrifugation at 15,000 rpm for 5 minutes diluted to an appropriate concentration with water by using Biotech Analyzer (Sakura Seiki) and Amino Acids Analyser L-8500 (HITACHI Keisokuki service). The L-arginine accumulation and yield at the point where the saccharide was depleted are shown in Table 3.

Table 3: L-arginine accumulation and yield of *arcA*-disrupted strain

Strain	L-arginine accumulation (g/L)	L-arginine yield (%)
237	4.04	6.73
2376ΔarcA	14.8	24.7

It was recognized that both of the accumulation and yield of L-arginine were improved in the *arcA* gene-disrupted strain compared with the control strain.